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# Tolerance-Inducing Passenger Leukocytes in Perinatal Skin Grafts of the Mouse

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**Neonatal skin grafts have the capacity to outlive adult skin grafts, especially in situations involving H-2 antigen compatibility of donor and host. Neonatal grafts can induce a state of tolerance in their hosts; in many cases, recipients exposed to neonatal allografts fail to reject adult grafts from the same donor strain.**

**In the H-2 antigen compatible C3H ↔ CBA combination, survival of neonatal skin grafts is correlated with the emigration of passenger leukocytes from the graft vasculature. Removal of a neonatal C3H skin graft has no effect on the unresponsive state of the host with respect to subsequent grafts of adult tissue. Donor leukocytes are present in various tissues of the tolerant CBA host, and treatments (such as irradiation) that eliminate the normal leukocytic population of a neonatal C3H skin graft also eliminate the likelihood of skin graft survival and block the establishment of tolerance.**

**In the C57BL/6 (B6) male-to-female system, survival of neonatal skin grafts is not correlated with emigration of passenger cells. Removal of a neonatal male B6 graft breaks the tolerance of the female host. The tolerant female is not a leukocyte chimera, and irradiation of the neonatal graft or replacement of the passenger cells with adult male cells does not eliminate the likelihood of graft survival nor does it block the establishment of tolerance. Moreover, passenger cells play no part in tolerance to Sk antigen incompatible grafts; Sk (skin-specific) antigens do not occur in lymphocytes.**

**The anomalous survival of neonatal skin grafts, evidently promoted by different mechanisms in different donor-host combinations, remains to be clarified. And it remains also to be determined whether the idiosyncratic performance of certain neonatal passenger cells has any physiological relevance.**

Genes that determine the presence of antigenic cell membrane components are called histocompatibility genes (H genes). The cell surface components are called histocompatibility antigens (in the mouse: H-1, H-2, H-3, etc.). A single "foreign" H antigen in a graft usually elicits in the host an immune response culminating in rejection of the graft. The speed of rejection is functionally related to the "strength" and number of H genes differing in donor and host. Weak genetic differences between donor and host are associated with chronic rejection, and strong differences with rapid rejection.

In every mammalian species there is a *major histocompatibility complex* (MHC), including genetic loci that determine formation of strong H antigens causing allograft reactions of maximal intensity. Consider, for example, the MHC of the mouse, H-2. Skin allografts exchanged between H-2 antigen incompatible donor and host almost invariably are rejected within 2 wk (often within 8 or 9 days). In contrast, grafts exchanged between animals with the same H-2 allele are rejected more slowly; these grafts may survive for 2 wk to more than 18 mo; the time of survival depends on the degree of "minor" immunogenetic disparity between the donor and host.

An unusual situation is encountered when the donor is a newborn or fetal animal (reviewed in reference 1). In cases of minor histoincompatibility, skin grafts from newborn or fetal donors often survive, while genetically "identical" grafts from adult donors are destroyed. In fact, perinatal grafts may render their hosts unresponsive to subsequent (or simultaneous) grafts of adult tissue.

In this article, I shall review a series of studies, performed in collaboration with my teacher, Willys K. Silvers of the University of Pennsylvania, showing that, in certain cases, the "privilege" extended to newborn or fetal grafts may be due to the emigration of passenger leukocytes from the graft vasculature.

## MATERIALS AND METHODS

The methods employed are standard; they are summarized below.

### *Animals*

We used the following inbred strains and their $F_1$ hybrids: A/Ss (A); CBA/Ss (CBA); C3H/HeJ (C3H); C57BL/6 (B6). Strain A mice are H-$2^a$, CBA and C3H mice are H-$2^k$, and B6 mice are H-$2^b$.

### *Transplantation*

Skin grafting was performed according to the method of Billingham [2]. Skin grafts from perinatal or adult donors were full-thickness disks or rectangles with areas representing about half the total integument of a newborn or late fetal donor. Starting 8 to 10 days after transplantation, we appraised graft survival by visual inspection, often with the aid of a dissecting microscope. The end point (rejection) was scored as the day of complete epithelial breakdown.

### *Statistics*

Median survival time (MST), standard deviation, and confidence limits were computed according to the method of Litchfield [3].

### *Irradiation*

In the B6 male-to-female and C3H ↔ CBA systems described below, irradiation was performed with a Standard X-ray machine (210 kvp; 15 ma; 0.5-mm Al filtration; 0.8-mm Cu half-value layer; and focus-to-skin distance of 60 cm at 160 R/min). In the skin-specific (Sk) antigen system, irradiation was performed with a $^{137}Cs$ source (Atomic Energy of Canada, Ltd) at about 120 R/min.

### *Cell Suspensions*

These were prepared according to procedures described elsewhere [4,5].

### *Induction of Tolerance*

Suspensions of $20 \times 10^6$ spleen and lymph node cells were injected into the orbital branches of the anterior facial veins [4] of neonatal mice (less than 18 hr old).

### *H-2 Typing*

In the Sk system, chimerism was verified serologically by the standard assays of hemagglutination (erythrocytes) and cytotoxicity (leukocytes) (as described in reference 6). Erythrocytes and leukocytes of A strain mice inoculated at birth with (A × B6)$F_1$ lymphocytes were typed positive for H-$2^a$ and H-$2^b$, thereby confirming the presence of H-$2^a$/H-$2^b$ cells of the donor type.

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Abbreviations:
MHC: major histocompatibility complex
MST: median survival time

TABLE I. *Influence of donor age on survival of B6 male-to-female skin grafts*[a]

| Donor | Number of recipients | Distribution of graft survival times (days) | | | | | | MST[b] (days) |
|---|---|---|---|---|---|---|---|---|
| | | < 14 | 15-24 | 25-50 | 51-75 | 76-100 | > 100 | |
| Adult | 101 | 5 | 46 | 45 | 4 | — | 1 | 25.0 [10]; 24.5 [11] |
| Neonatal | 77 | — | 6 | 9 | 6 | 3 | 53 | (69% "tolerant") |

[a] Combined data from Billingham, Silvers, and Wilson [10] and Silvers [11] showing extended survival of neonatal male skin "isografts" on B6 female recipients.
[b] Abbreviation: MST, median survival time.

## RESULTS AND DISCUSSION

### *Neonatal Skin Grafts: The H-Y System*

In certain highly inbred mouse strains, especially those with the H-$2^b$ allele, male tissues are incompatible in female recipients. For example, in the B6 strain, male-to-female skin grafts are uniformly rejected, whereas skin grafts exchanged between the other sex combinations (male-to-male, female-to-male, female-to-female) are uniformly accepted [7]. Rejection in this case is due to presence in donor tissues of H-Y antigen, a male-specific cell surface component determined either directly or indirectly by genes on the Y chromosome. (H-Y antigen is widely conserved among vertebrates, and there is now a persuasive body of evidence that the serologically detectable molecule is the inducer of the mammalian testis [see discussion in reference 8].)

H-Y has been classified as a "weak" antigen; the MSTs of H-Y-incompatible skin grafts tend to cluster around 25 days; the time depends on the size of the grafts and on the site from which the donor skin was excised [9].

In the 1965 study of Billingham, Silvers, and Wilson [10], the MST of 71 male B6 skin grafts was 25 days on B6 females; in a 1968 study [11], the MST of 30 such grafts was 24.5 days. But the situation was radically different when the grafts came from *neonatal* donors (less than 24 hr old). In the 1965 study, 19 of 31 grafts from newborn males (61%) were "tolerated" (i.e., survived throughout the 100-day observation period), and in the 1968 study, 34 of 46 grafts from newborn males (75%) were tolerated (Table I). It is unlikely that tolerance was due to the absence of H-Y antigen in the grafted perinatal tissues. In both studies, grafts borne by adult females for more than 100 days were rejected when removed and retransplanted to normal female hosts, though rather more slowly than "fresh" adult male skin isografts (the MST of 23 retransplanted grafts was 36 days; 2 survived more than 100 days on the secondary hosts [11].) Moreover, if it were absence of H-Y antigen that allowed long-term survival of neonatal male skin grafts on adult female hosts, one might expect *adult* male skin grafts transplanted contralaterally to be rejected by the female host with normal or near normal vigor. But failure of the B6 female host to reject neonatal male skin isografts was due to a state of unresponsiveness induced in the host by the neonatal tissues. In the studies cited above, when B6 females bearing neonatal male skin isografts for more than 50 days were challenged on the other side of the thorax with adult male skin isografts, fully half of the hosts tolerated both grafts. When B6 females were exposed simultaneously to contralateral adult and neonatal male skin isografts, 23% of the hosts tolerated both grafts. So it seems that neonatal male skin grafts release antigen in a "tolerance-inducing" form.

### *Neonatal Skin Grafts: The C3H ↔ CBA System*

C3H and CBA mice are derived from a common stock. Both strains are compatible with respect to the MHC; both are H-$2^k$. Yet these strains differ with respect to several H genes.

We found that skin grafts from adult CBA mice were rejected by C3H recipients in 12.2 days. Skin grafts from neonatal CBA mice were also rejected in 12.2 days [12]. However, when C3H hosts were exposed to whole-body X-irradiation, a treatment known to depress immunologic reactivity, the MST of the neonatal CBA skin grafts was increased considerably relative to the MST of the adult skin grafts (Table II). At 100 R, the MST of the neonatal grafts was increased to 16.1 days, but the MST of adult grafts was not increased. At 400 R, the neonatal grafts survived for 41 days; the life of the adult grafts was increased only slightly, to 21 days (Fig. 1).

TABLE II. *Survival of CBA adult and neonatal skin grafts on adult male and female C3H recipients exposed to X-rays*[a]

| X-ray dose (R) | Donor | No. mice | MST[b] (days) | ± 95% confidence limits (days) |
|---|---|---|---|---|
| None | Adult | 21 | 12.2 | .8 |
| | Neonate | 21 | 12.2 | .6 |
| 100 | Adult | 10 | 12.5 | .9 |
| | Neonate | 11 | 17.0 | 1.2 |
| 200 | Adult | 12 | 16.1 | 1.3 |
| | Neonate | 15 | 20.3 | 2.1 |
| 300 | Adult | 12 | 17.2 | 1.0 |
| | Neonate | 22 | 26.2 | 3.0 |
| 400 | Adult | 13 | 20.8 | 2.2 |
| | Neonate | 16 | 41.0 | 5.0 |

[a] See Fig 1 and reference 12.
[b] Abbreviation: MST, median survival time.

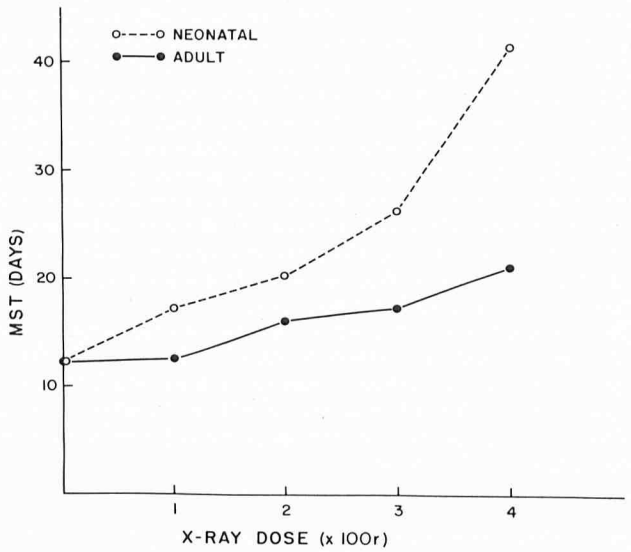


FIG 1. Median survival times (*MST*) of neonatal and adult CBA skin grafts on panels of C3H mice that had received graded doses of whole-body X-irradiation (see Table I). Taken from Wachtel and Silvers [12]. Reprinted by permission: *The Journal of Experimental Medicine.*

The disparity between neonatal and adult graft survival was striking when CBA mice were used as recipients. The MST of adult C3H grafts on 28 CBA recipients was only 16.0 days, but many of the C3H neonatal skin grafts survived permanently: 4 of 19 (21%) on CBA female hosts and 21 of 27 (78%) on CBA male hosts. (Differences in the immunologic reactivity of males and females, generally "favoring" females, are known in several systems [reviewed in reference 1].) Moreover, the neonatal grafts were able to induce a state of unresponsiveness to adult C3H skin grafts transplanted 100 days later. Of the 16 CBA male recipients that had accepted neonatal C3H skin grafts, all

now tolerated adult C3H grafts. In contrast, the 4 CBA females that had accepted neonatal grafts now rejected the adult 2nd grafts, after 9, 10, 16, and 21 days (Table III).

Gene dosage effects are recognized for several systems of cell surface antigens. Representation of a cell surface antigen on heterozygous cells seems to be less than that on corresponding homozygous cells (see, for example, references 13,14). Accordingly, we next grafted (heterozygous) skin from neonatal (CBA × C3H)$F_1$ hybrid mice onto females of the CBA strain. (We have seen that 79% of these females reject [homozygous] skin grafts from neonatal C3H donors.) Of 26 female CBA mice that received (CBA × C3H) neonatal skin grafts, 21 (81%) accepted the grafts; 20 of the 21 accepted subsequent adult C3H grafts after the neonatal grafts had been in place for 100 days. Thus, as in the H-Y system, neonatal skin grafts survive longer than adult grafts in the CBA ↔ C3H strain combination, the latter involving multiple non-H-2 disparities. In the C3H → CBA direction, neonatal skin grafts may induce in their hosts a state of unresponsiveness to subsequent (or even simultaneous) adult skin grafts. In some situations in which homozygous grafts are rejected, heterozygous ($F_1$) grafts may persist and likewise induce unresponsiveness to parental strain adult skin grafts.

*Neonatal Skin Grafts: The Sk System*

The Chimaera is a mythological monster, often depicted as a composite of lion, goat, and serpent. The chimera of biologists is less awesome; it merely represents genetically distinct populations of cells within the same organism. In immunology, a *radiation chimera* is an animal (for instance, a mouse) that has been irradiated (to eliminate immunologically competent host lymphoid cells and their precursors) and then "restored" with an inoculum of hematopoietic cells from another (genetically disparate) animal. A *neonatal chimera* is an animal that has been exposed at birth or shortly thereafter to an inoculum of genetically disparate lymphohematopoietic cells. In both situations, the immunologist creates in the host a state of immunologic "inefficiency" with respect to donor cells—in the former case by eliminating immunocompetent (radiosensitive) cells, and in the latter case by selecting immature animals unable, by virtue of their age, to reject donor cells (sublethal doses of irradiation are sometimes employed in establishing neonatal chimerism). Thus, chimeric animals contain immunocytes of donor origin, and, when the genetic combination is such that the donor cells cannot react against host antigens ($F_1$ → parental), the result is mutual tolerance. It follows that skin grafts from an (A × B)$F_1$ donor should survive indefinitely in B hosts lethally irradiated and reconstituted with (A × B)$F_1$ bone marrow cells, or in B hosts inoculated "neonatally" with (A × B)$F_1$ bone marrow cells, as long as the chimerism is maintained.

Yet hematopoietic chimerism does not ensure acceptance of skin grafts from the donor of the colonizing cells (reviewed in references 15,16). For example, B6 mice lethally irradiated and then reconstituted with bone marrow cells, spleen cells, or both from (B6 × A) or (A × B6)$F_1$ donors often reject subsequent A, (B6 × A)$F_1$, or (A × B6)$F_1$ skin grafts, even though the recipients are chimeric with respect to erythrocytes and leukocytes of donor origin [15]. Rejection in this system is due to a tissue-specific differentiation alloantigen (Sk) present in skin (and neurectodermal derivatives such as brain) but absent in lymphoid cells. Thus, lymphocytes removed from an environment in which a particular Sk antigen is expressed gain the capacity to react against it and thereby exhibit a manifestly autoimmune reaction. There is, of course, the possibility that rejection of Sk antigen incompatible skin grafts in radiation chimeras is due to survival of a residuum of radiation-resistant hematopoietic stem cells of the *host*, but this possibility should have no bearing on the selective expression of Sk antigen since the surviving host cells should be tolerant of antigens borne by colonizing lymphocytes of the donor in any event. Indeed, Sk antigens are also indicated in cases of neonatal chimerism in which donor and host cells coexist, for example in B6 mice subjected to sublethal doses of X-irradiation (400 to 500 R) and then inoculated within 24 hr of birth with $20 \times 10^6$ lymphocytes from adult (A × B6)$F_1$ donors. In the study of Silvers et al [6], these neonatal chimeras subsequently rejected skin grafts (transplanted 12 to 14 wk after birth of the host) from A strain adult donors, despite persistent hematopoietic chimerism, demonstrable by H-2 typing in erythrocytes and leukocytes. However, the chimeric hosts failed to react with equal vigor when *neonatal* A strain skin grafts were used instead of adult skin grafts. Although adult grafts were rejected uniformly, 31% of the neonatal grafts were accepted by chimeric females, and 64% by chimeric males (Table IV).

In view of the earlier observations in B6 mice and in C3H and CBA mice, it was not surprising to find that Sk antigen incompatible grafts from neonatal donors could induce in their hosts a state of unresponsiveness to subsequent Sk antigen incompatible grafts of adult origin. Of 10 chimeric B6 ← (A × B6)$F_1$ mice bearing long-term neonatal A strain skin grafts and challenged with adult A strain skin grafts, 8 permanently accepted both.

*Passenger Cells: The H-Y and Sk Systems*

In 1967, David Steinmuller [17] showed that A skin *isografts* from A ← (A × C3H) neonatal chimeras, i.e., from A mice carrying (A × C3H)$F_1$ lymphoid cells, could immunize A strain recipients against subsequent C3H skin *allografts* carrying antigens in common with the (A × C3H)$F_1$ inoculum. He deduced that (A × C3H) lymphoid cells borne passively within the vasculature of the A → A skin isografts were immunogenic (see discussion by Steinmuller [18] in this issue). In the literature there are numerous reports consistent with this view (reviewed in references 18,19), and indeed it is widely accepted that "contaminating" passenger leukocytes not only serve as a source of sensitizing antigen, but also contribute to the susceptibility of solid-tissue allografts to the host's immune response.

Are passenger leukocytes involved in the privileged survival of neonatal skin grafts? Certainly not in the Sk system; by definition Sk antigen is not found on the leukocyte membrane. Specific tolerance (generally representing extraordinary expo-

TABLE III. *Survival of adult and neonatal C3H and $F_1$ skin grafts on CBA recipients*[a]

| Donor | Recipient | | No. (%) tolerant | MST[b] (days) | ± 95% confidence limits (days) |
|---|---|---|---|---|---|
| | Sex | No. | | | |
| C3H adult | Male and Female | 28 | — | 16.0 | 1.4 |
| C3H Neonatal | Male | 27 | 21 (78)[c] | — | — |
| | Female | 19 | 4 (21)[d] | 17.0 | 3.1 |
| (CBA × C3H)$F_1$ Adult | Male and Female | 21 | 1 (5) | 19.0 | 1.8 |
| (CBA × C3H)$F_1$ Neonatal | Female | 26 | 21 (81)[e] | — | — |

[a] See reference 12.
[b] Abbreviation: MST, median survival time.
[c] Of these recipients, 16 were challenged with an adult C3H skin graft after 100 days; all 16 tolerated both grafts.
[d] None of these recipients accepted a subsequent adult C3H skin graft.
[e] Of these recipients, 20 accepted a subsequent adult C3H skin graft.

TABLE IV. *Influence of donor age on survival of Sk antigen-incompatible A strain skin grafts on B6 ← (A × B6)F$_1$ chimeric mice*[a]

| Donor | Recipient | | Range of graft survival times (days) | MST[b] (days) | % hosts tolerant |
|---|---|---|---|---|---|
| | Sex | No. | | | |
| Adult | Male | 16 | 13–69 | 24.0 | — |
| | Female | 13 | 13–45 | 13.2 | — |
| Neonatal | Male | 22 | 15–>100 | — | 64 |
| | Female | 13 | 15–>100 | — | 31 |

[a] Recipients irradiated (500 R) and inoculated at birth with $20 \times 10^6$ (A × B6)F$_1$ lymphoid cells. Note the difference in the responses of males and females; compare with data in Table III. Taken from Silvers, Wachtel, and Pool [6].

[b] Abbreviation: MST, median survival time.

sure of the host to antigen) requires, for its persistence, the persistence of antigen [20]. If it were passenger leukocytes that induced tolerance to a particular skin graft, removal of the graft should not affect the tolerant state; on the other hand, if it were the intact graft itself that induced tolerance, removal of the graft should "break" the tolerant state. It follows that complete removal of Sk antigen incompatible neonatal skin grafts before exposure of the host to a secondary adult graft should result in destruction of the adult grafts. Silvers et al [6] carefully removed 6 neonatal (Sk antigen incompatible) A skin grafts after they had been in place for 200 days on their B6 chimeric male hosts. When challenged 50 days later with adult A skin grafts, the hosts reacted as if they had never "seen" the neonatal grafts. The adult grafts were all rejected, and the range of survival times (20 to 47 days) approximated that (13 to 69 days) observed for similar grafts on naive chimeric male hosts.

A similar situation was observed in the B6 male-to-female system. Excision of long-surviving neonatal B6 male grafts also resulted in loss of tolerance. When their neonatal male grafts of 100 days' standing were removed, 20 adult B6 female hosts rejected adult B6 male skin grafts (transplanted 50 days later) in an *accelerated* fashion (a single adult male graft survived for >100 days [11]). This phenomenon indicated that survival of neonatal B6 male skin grafts was not due to the presence of passenger leukocytes. To examine the question further, we performed several tests [5]:

*Irradiation of the graft:* If passenger cells are critical to the extended survival of neonatal male B6 skin grafts, selective destruction of the passenger cells should prejudice graft survival. We have pointed out that 850 R of X-irradiation are lethal for hematopoietic cells. Accordingly, skin from neonatal B6 male mice exposed to 850 R of whole-body X-irradiation was grafted onto a panel of 23 adult B6 females. Skin from irradiated B6 neonatal females served as controls. All of the female grafts survived permanently on the B6 female hosts; 65% of the male grafts survived permanently; of these, half induced tolerance to subsequent adult male grafts.

*Emigration of passenger cells:* There is evidence of extensive vascular continuity between skin graft and host within 48 hr of transplantation [21]. Within 4 days the circulating lymphocyte moiety of a newborn skin graft should therefore be replaced by adult cells of host origin. We transplanted neonatal male B6 skin onto adult male B6 mice and allowed the grafts to remain in place for 4 days. At the end of that time, the grafts were removed, carefully trimmed, and retransplanted onto adult female B6 recipients. The procedure did not influence the survival of the neonatal male skin grafts (presumably containing adult leukocytes; see below). Of 14 grafts so treated, half were accepted permanently, whereas 7 of 12 untreated skin grafts from 4-day-old males were accepted permanently (58%).

*Analysis of chimerism:* Adult B6 females bearing long-term neonatal male B6 skin grafts evidently are not chimeric. Tissues from these tolerant females do not detectably influence the response of secondary female hosts to adult male skin grafts. With a modified version of the method of Billingham, Silvers, and Wilson [10], we exposed B6 adult females to isografts of various tissues from B6 females tolerant of neonatal male skin grafts, and then to test grafts from adult B6 males. The results (Table V) failed to indicate clearly the presence of male lymphoid cells in these tissues. (It is perhaps worth pointing out that in neonatal female B6 mice contamination of an inoculum of 20 million B6 female cells with 12,000 male cells produces a state of tolerance to H-Y antigens in the hosts [10].)

*Direct inoculation of leukocytes:* The results described in the preceding paragraphs argued against the view that the survival of neonatal male B6 skin grafts depends on passenger cells, but they did not rule out the possibility that passenger cells may contribute to the induction of unresponsiveness in females bearing neonatal male grafts. We therefore injected from 1 to 6 million cells from neonatal B6 male thymus or peripheral blood and from 16-day-old fetal B6 male liver subcutaneously into B6 females; after 50 days, we challenged the females with adult male skin isografts. In all cases the adult male grafts were rejected, generally in an *accelerated* fashion (Table VI). Far from rendering their hosts tolerant, the perinatal male leukocytes had sensitized them. Thus, in the B6 male-to-female system, passenger leukocytes appear to make little or no contribution to the long-term survival of neonatal male skin grafts, or to the induction of tolerance to subsequent adult male skin grafts. The situation is radically different, however, in other donor-host combinations.

### *Passenger Cells: The C3H ↔ CBA System*

Earlier I mentioned that the maintenance of specific tolerance depended on the persistence of antigen, and that removal of Sk or H-Y incompatible neonatal grafts caused loss of tolerance in their hosts. In contrast, removal of the neonatal C3H graft did not cause loss of tolerance in CBA hosts. We removed C3H neonatal skin grafts of 100 days' standing from 12 adult CBA males and, after 50 days, challenged each with an adult C3H graft [5]. Of the adult grafts, 2 were rejected in an accelerated fashion and 2 were rejected on days 34 and 36; 8 were not rejected.

It seems that the unresponsiveness of CBA mice exposed to neonatal C3H skin grafts is related to the emigration of passenger leukocytes from the graft vasculature; tolerant CBA mice are CBA ← C3H leukocyte chimeras. This conclusion is borne out by evidence from several experiments [5].*

*Irradiation of the graft:* When newborn C3H mice were exposed to 850 R of whole-body X-irradiation just before grafting, skin from these animals did *not* survive on adult CBA recipients, but skin from irradiated newborn C3H mice *did* survive on adult C3H (isogenic) recipients. In fact, all of 15 isografts survived permanently, whereas only 1 of 19 (5.3%) allografts survived permanently (compare these figures with the 78% permanent survival of untreated C3H neonatal skin allografts on CBA males). These data suggest that passenger cells in the C3H ↔ CBA combination do contribute to the survival of neonatal C3H grafts unlike passenger cells in the B6 male → B6 female combination. Yet it could be argued that

* In most of these experiments, C3H mice were used as donors and CBA mice were used as recipients because tolerance could be readily induced in that direction with neonatal grafts. We primarily used CBA male recipients because they are less likely than females to reject such grafts.

TABLE V. *Survival of adult male B6 skin grafts on female B6 mice exposed to isografts from tolerant B6 females*[a]

| Isograft tissue[b] | No. of hosts | MST ± SD (days)[c] |
|---|---|---|
| Lymph node | 9 | 22.0 ± 1.3 |
| Spleen | 7 | 20.0 ± 1.3 |
| Thymus | 10 | 22.5 ± 1.2 |
| Bone marrow | 4 | 27.0 ± 1.2 |
| Skin | 18 | 22.7 ± 1.2 |

[a] See reference 5.
[b] With the exception of skin, $40 \times 10^6$ cells from each tissue of B6 females bearing neonatal B6 male skin grafts for more than 100 days were injected intraperitoneally into naive B6 females; the recipients were challenged 7 days later with skin from adult B6 males.
[c] Control median survival time (MST), 24.5 days.

TABLE VI. *Effect of subcutaneous inoculation of B6 male fetal, neonatal, or adult leukocytes on the survival of adult male B6 skin grafts*[a] *in B6 adult females*[b]

| Type cells injected | No. cells injected ($\times 10^6$) | No. hosts | MST[c] ± SD (days)[c] |
|---|---|---|---|
| Neonatal thymus | 5 | 8 | 11.0 ± 1.2 |
| Adult thymus | 5 | 11 | 10.0 ± 1.1 |
| Neonatal leukocytes | 1 | 20 | 16.3 ± 1.6 |
| Adult leukocytes | 1 | 12 | 14.5 ± 1.5 |
| Fetal liver[d] | 6 | 12 | 10.3 ± 1.1 |
| Adult liver[d] | 6 | 6 | 13.2 ± 1.2 |

[a] Transplanted 50 days after inoculation.
[b] See reference 5.
[c] Abbreviation: MST, median survival time.
[d] Liver from a 16-day-old male fetus.

failure of neonatal allograft survival in these experiments was not due to elimination of passenger cells, but to a synergistic effect of X-irradiation in combination with the normal immune response of the host. (Skin allografts from irradiated C3H adults also fared poorly on CBA hosts.) We therefore arranged for a less traumatic elimination of graft-borne leukocytes (see next paragraph).

*Emigration of passenger cells.* Skin from 17-day-old C3H fetuses was isografted to adult C3H mice, allowed to remain in place for 4 days, and then removed, cleaned, and allografted to a panel of adult male CBA mice. The rationale for these experiments was the same as for similar experiments described above: vascular connections between tissues of the isograft and primary host should promote exchange of blood. Consequently, the "neonatal" grafts were expected to carry *adult* passenger cells to the secondary allogeneic host. If passenger cells were critical to the life of the neonatal allograft, rejection should ensue. The stratagem worked. Of 24 secondary hosts, only 1 (4.2%) was unresponsive; the MST of the perinatal C3H grafts was 15.1 days (compare this figure with the 16.0-day MST for adult C3H skin grafts on CBA recipients). If fetal skin grafts acquire circulating leukocytes of the primary host, do these cells pass into the tissues of the secondary host? Consider an experiment (Fig 2) modeled after the system described in [17]. Panels of A strain mice were made chimeric through injections, given at birth, of spleen and lymph node cells from adult (C3H × A)$F_1$ donors. When they were 3 to 6 months old, the A←(C3H × A) chimeras were used as primary hosts for skin isografts from 17-day-old fetal A strain donors. After 4 days, the grafts were excised and retransplanted (isografted) to a panel of 17 hitherto untreated A strain adults. Seven days later, each A strain adult received an allograft of C3H skin on the other side of its thorax. The C3H grafts were sloughed in accelerated fashion. The 7.8-day MST for these C3H grafts was significantly less than the 10.7-day MST for similar grafts on "naive" A strain adults. It seems that the secondary hosts were immunized against C3H antigens by exposure to passenger cells acquired in the perinatal isografts. By inference, loss of neonatal passenger leukocytes is inconsistent with survival of neonatal C3H grafts on CBA hosts.

*Analysis of chimerism:* To determine whether C3H neonatal passenger cells colonize CBA hosts, we exposed female adult CBA mice to isografts of various tissues from CBA mice bearing neonatal C3H skin grafts for more than 100 days, and then to skin grafts from C3H adults. We thought the presence of colonizing C3H cells in the CBA isografts would immunize the CBA hosts against C3H antigens; this immunization would be manifest as a "second-set" (accelerated) rejection of the C3H test grafts. Indeed, the C3H test grafts were rejected acutely (Table VII), an indication that their hosts had been immunized by isografts putatively bearing C3H passenger cells. Yet the

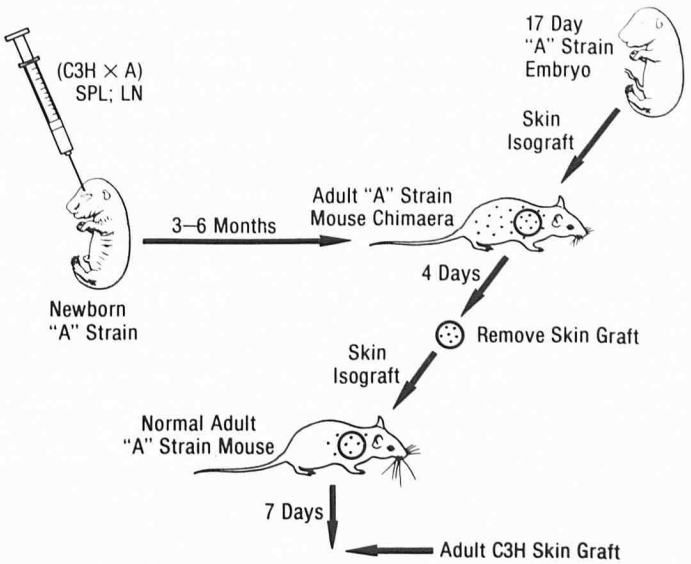


FIG 2. Experimental protocol for detection of acquired "chimeric" passenger cells in a perinatal A strain skin graft [5, 17]. *SPL*, spleen; *LN*, lymph node.

TABLE VII. *Survival of adult C3H skin grafts on adult CBA mice exposed to isografts from tolerant CBA mice*[a]

| Isograft tissue[b] | No. hosts | MST ± SD (days)[c] |
|---|---|---|
| Lymph node | 8 | 7.5 ± 1.3 |
| Spleen | 11 | 7.5 ± 1.1 |
| Thymus | 8 | 9.0 ± 1.4 |
| Skin | 22 | 11.2 ± 1.3 |

[a] Bearing neonatal C3H skin grafts > 100 days. See reference 5.
[b] Cells ($40 \times 10^6$) of designated tissue (except skin) from tolerant CBA males were injected intraperitoneally into naive CBA male and female recipients; the recipients were challenged 7 days later with skin from adult C3H mice.
[c] Control median survival time (MST), 16.0 days (see Table III).

TABLE VIII. *Effect of subcutaneous inoculation of C3H fetal, neonatal, or adult leukocytes on the survival of C3H adult skin grafts*[a] *in adult CBA recipients*[b]

| Type cells injected | No. cells injected ($\times 10^6$) | No. hosts | MST[c] ± SD (days) | No. (%) hosts tolerant |
|---|---|---|---|---|
| Neonatal thymus | 5 | 16 | 11.0 ± 1.3 | 1 (6) |
| Adult thymus | 5 | 11 | 10.0 ± 1.1 | — |
| Neonatal leukocytes | 1 | 19 | 12.0 ± 1.6 | 2 (11) |
| Adult leukocytes | 1 | 12 | 9.1 ± 1.1 | — |
| Fetal liver[d] | 1 | 6 | 10.0 ± 1.0 | — |
| Adult liver | 1 | 6 | 9.4 ± 1.1 | — |
| Neonatal spleen | 1 | 12 | 10.0 ± 2.7 | — |

[a] Transplanted 50 days after inoculation.
[b] See reference 5.
[c] Abbreviation: MST, median survival time.
[d] Liver from 14- to 15-day-old fetuses.

data may have been an indication that antigens were sequestered in CBA *isologous* passenger cells in such a way that presentation to the secondary hosts rendered them immune. One might argue, moreover, that accelerated rejection in this system reflected passive transfer of immune cells from tolerant animals [22], although in other tests we could not transfer immunity into naive CBA mice by exposing them to $40 \times 10^6$ thymus cells or to skin grafts from sensitized CBA mice, i.e., CBA mice that had rejected C3H skin grafts [5].

*Direct inoculation of leukocytes:* To determine whether leukocytes acting alone can induce tolerance in the C3H ↔ CBA system, we exposed adult CBA mice to subcutaneous injections of leukocytes from the thymuses, peripheral blood, and spleens of neonatal C3H mice and from the livers of fetal C3H mice; the recipients received grafts of adult C3H skin 50 days after inoculation. As shown in Table VIII, CBA males exposed to leukocytes of C3H adult, neonatal, or fetal origin were generally sensitized and rejected their C3H test grafts in an accelerated fashion; 3 CBA males failed altogether to reject the grafts. The fact that unresponsiveness was secondary to exposure to circulating leukocytes in 2 cases indicates that these cells need not be carried in a skin graft to influence the host (though they are evidently more efficient "tolerance inducers" when neonatal skin grafts serve as their vehicle).

The results described above are consistent with the view that passenger cells may influence the immune response of the host toward solid-tissue grafts and with the view that these cells may actually promote a state of tolerance in certain combinations involving neonatal tissues. Thus, the extended survival of neonatal C3H skin grafts seems dependent on survival and emigration of the leukocyte moiety. It is not clear whether passenger leukocytes also influence the extended survival of reciprocal CBA neonatal skin grafts exhibited in irradiated C3H recipients.

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